Chromatin Remodeling Agents for Cancer Therapy

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Abstract: Alterations in chromatin structure profoundly influence gene expression during normal cellular homeostasis and malignant transformation. Methylation of cytosines within CpG islands located in promoter and proximal coding regions facilitates recruitment of chromatin-remodeling proteins, which inhibits gene expression. Posttranslational modifications, such as acetylation, methylation, and phosphorylation, of core histone proteins ''mark'' regions of chromatin for recognition by multiprotein complexes, which promote either chromatin relaxation and gene expression or chromatin compaction and repression of gene expression. Many genes become transcriptionally silenced during the development of cancer. Covalent epigenetic modifications such as DNA hypermethylation and histone post-translational modifications are an important early event during carcinogenesis and tumor development. Genes involved in key DNA damage responses pathways, apoptosis signaling and DNA repair, can frequently become methylated and epigenetically silenced in tumors. This may lead to differences in intrinsic sensitivity of tumors to chemotherapy, depending on the specific function of the gene inactivated. The fact that cancer can have an epigenetic etiology has encouraged the development of a new therapeutic option that might be termed "epigenetic therapy". The DNA methylation paradox, manifested as derepression of cancer-testis antigens and silencing of tumor suppressors during malignant transformation, provides rationale for the utilization of chromatin remodeling agents for cancer therapy. In this review, the recent advances in the understanding and clinical development of DNA methyltransferase and Histone deacetylase inhibitors, as well as their current role in cancer therapy, will be discussed.

Keywords: Cancer, epigenetic therapy, demethylating agents, deacetylating agents.

INTRODUCTION

DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function. Epigenetic modifications, which include covalent modification of bases in the DNA and of amino-acid residues in the histones, are generally stable and heritable in somatic differentiated cells. The methylation profile of the cell is exquisitely controlled during development. In germ cells and in pre-implantation embryos there are at least two developmental periods in which methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Epigenetic reprogramming is critical and affects the imprinting in germ cells and early embryos [1]. Reprogramming is likely to have a crucial role in establishing nuclear totipotency in normal development, in stem cell differentiation and in ensuring acquired epigenetic information. The existence of an epigenetic-based cellular memory, or program, serves to regulate global patterns of gene expression and is the basis of genome defence mechanisms that silences viruses and trasposons [2]. The engines of epigenetic change in mammals are the DNA methylation, a chemical modification to DNA, and changes in chromatin structure resulting from histone modifications. These DNA chromatin modifications are potentially reversible and modulate gene expression without changing DNA sequence and without any new genetic information. Chromatin remodeling by nucleosome reorganization at the site of the promoter genes enables transcriptional regulation. Histone acetylation and DNA methylation are the two best characterized epigenetic modifications to which histone methylation, phosphorylation and ubiquitination must be added. Histone acethyla tion and DNA methylation are interdependent and their equilibrium contributes to regulate gene expression [3]. CpG methylation appears to differ from histone modifications because it bestows a persistent epigenetic memory independently of histone modifications, which appears to be limited in this capacity. Within the DNA methylation changes there are regional specific differences in the methylation pattern. The genome appears to be compartmentalized with respect to methylation of CpG sites. The methylated compartments appear to coincide with the regions of gene inactivity whereas the unmethylated compartments coincide with regions of gene activity. Repeated sequences which comprise up to 35% of the genome are generally hypermethylated while the CpG sites within the promoter region and first exon of house-keeping genes and of tissue-specific genes are unmethylated [4].

In contrast to normal cells, the methylation pattern in cancer cells is disrupted with the major changes in the methylation compartments of the cells. The normally hypermethylated and silenced regions containing repetitive elements and oncogenes are demethylated while unmethylated CpG island containing tumor suppressor genes often become hypermethylated and inactivated [5, 6]. In cancerous cells, the total content of methyl cytosine is reduced by about 40% and it has been proposed that this hypomethylation contribute to malignancy by either contributing directly to the acti-

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vation of oncogenes, activation of latent retrotrasposons and /or chromosome instability [7]. However, there is also a marked increase in the methylation of specific genomic regions, called CpG islands. These regions are located within the vicinity of promoters for genes that carry out basic functions of cells. Based on research efforts to understanding the impact of epigenetic on cancer, it has been established that an excessive methylation of tumor suppressor genes is a common hallmark of all human cancer. Genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, apoptosis, angiogenesis and metastasis have all been identified as being susceptible to hypermethylation in different cancer cells [8]. The fact that cancers have an epigenetic etiology has encouraged the development of a new therapeutic option that might be termed "epigenetic therapy". The DNA methylation paradox, manifested mostly as silencing of tumor suppressors during malignant transformation, provides the rationale for the utilization of chromatin remodeling agents for cancer therapy.

INSIGHTS ON MOLECULAR MACHINERY FOR EPIGENETIC GENE SILENCING

The best studied epigenetic modifications are DNA methylation and post-translational histone modifications. DNA methylation is the covalent addition of a methyl group to the DNA, predominantly to the base cytosine 5' to guanine, also called CpG dinucleotide. DNA methylation is catalysed by DNA methyltransferases (DNMTs), of which three active enzymes have been identified in mammals, namely DNMT1, DNMT3a and DNMT3b. The DNMT1 is responsible for maintaining the pre-existing methylation patterns following DNA replication, while DNMT3a and DNMT3b are required for initiation of *de novo* methylation [9, 10]. Several evidences indicate that the three DMNTs not only cooperate but also may process both *de novo* and maintenance functions *in vivo* [11, 12]. The methylated DNA is in part interpreted by methyl-binding domain proteins (MBDs), including MeCP2 and MBD 1-4. These MBD proteins are important "translators" between DNA methylation and histone-modifier genes that establish a transcriptionally inactive chromatin environment. Most of hypermetylated promoters are occupied by a particular set of MBD proteins, which seem to be gene and tumor-type-specific. The treatment of cancer cells with demethylating agents causes CpG island demethylation, MBD release and gene re-expression, reinforcing the notion that association of MBDs with methylated promoters is methylation-dependent. The finding that MeCP2 repress transcription of methylated DNA through the recruitment of histone deacetylase-containing complex, establishes a mechanistic connection between DNA methylation and transcriptional repression by the modification of chromatin. Chromatin remodeling by nucleosome reorganization at the site of promoter genes enables transcriptional regulation [13]. At the nucleosome level the DNA helix is wrapped around the histone proteins, which are subjected to various modifications as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, glycosylation, biotinylation and carbonylation. The totality of histone modifications, or "histone code", is read by proteins involved in chromatin remodeling, transcriptional activation or repression, and thereby govern chromatin dynamics and gene transcription. In particular, histone acetylation, which is controlled by histone acetyltransferases (HATs), generally correlates to an open and transcriptionally active chromatin state, whereas histone deacetylation, controlled by histone deacetylases (HDACs), is associated with chromatin condensation and transcriptional repression. Thus, hypermethylated CpG islands of silenced tumor suppressor genes are known to display a histone code or post-translational modifications characterized by histone hypoacetylation and histone methylation [14]. A close interconnection between DNA and histone modifications has been found in gene silencing. DNMTs are able to recruit HDACs, and both DNMTs and MBDs recruit histone methyltransferases (HMTs) that modify lysine 9 of histone H3 [15]. In the hypermethylated promoters this active recruitment of multiple repressors leads to a characteristic histone modification pattern featuring deacetylation of histone H3 and H4, methylation of lysine 9 of histone H3, and demethylation of lysine 4 of histone H3 [16]. Another connection between various repression systems is highlighted by Polycomb proteins. These proteins are involved in silencing "master genes" involved in major developmental processes and are found to cooperate with DNMTs and MBD proteins to establish DNA methylation in a subset of target genes [17, 18].

Thus, among the multitude of proteins which crowd the promoter of epigenetically silenced genes, it is clear that some proteins, such as MBDs, are more permanent residents and other, such as DNMTs, HDACs, HMTs and Polycomb, intermix to maintain a chromatin conformation compatible with stable epigenetic transcriptional silencing.

ROLE OF DNA HYPERMETHYLATION AND HISTONE DEACETYLATION IN THE PATHOGENESIS OF CANCER

1. Aberrant DNA Methylation and Cancer Development

Cancer is a stepwise process of accumulation of genetic and epigenetic abnormalities that can lead to cellular dysfunction and the synergy of these two processes drives tumor progression and malignancy. The most emphasized alteration of DNA methylation in cancer is the aberrant hypermethylation of CpG islands surrounding promoter regions [19]. A growing number of tumor-suppressor and other cancer-related genes have been demonstrated to be silenced by aberrant promoter methylation. *De novo* methylation of 'CpG islands' in the promoter regions of tumor suppressor genes may lead to transcriptional silencing through a complex process involving histone deacetylation and chromatin condensation, and thus represents a tumorigenic event that is functionally equivalent to genetic changes, like mutation and deletion [20]. The genes affected include over half of the tumor suppressor genes that cause familial cancers when mutated in the germ line. The selective advantage for genetic and epigenetic dysfunction in these genes is very similar. For some genes, the promoter methylation may be the only type of gene inactivation found in human cancer, since mutations for many of genes are rare or have not been observed. The aberrant methylation can begin very early in tumor progression and mediate most of the important pathway abnormali-

ties in cancer including loss of cell cycle control, altered function of transcription factors, altered receptor function, disruption of normal cell-cell and cell-substratum interactions, inactivation of signal transduction pathways, loss of pro-apoptotic signals and genetic instability. The profile of gene promoter hypermethylation differs for each cancer type, providing a tumor-type and gene-specific profile. This is consistent with a model in which methylation of CpG islands at particular genes would give to the cancer cell a growth or survival advantage and so aberrant patterns of methylation emerge depending on the selective pressure for gene silencing in the tumors type examined [21]. Some genes, such as the cell cycle inhibitor $p16^{INK4a}$, are hypermethylated across many tumor types including colorectal, lung, and breast carcinomas. This alteration reflects the widespread contribution of disruptions of the cyclinD-Rb cell cycle control pathway in human cancer [22]. Other changes, such as for the DNA repair gene MGMT and DAPK, also have a wide distribution [23]. Hypermethylation of $p14^{ARF}$ and APC are most prevalent in gastrointestinal tumors (*i.e.*, colon and stomach) [24], whereas GSTP1 is characteristic of steroid-related neoplasm such as breast, liver, and prostate [25, 26]. The aberrant methylation of certain genes reflects their very specific involvement in selected tumor types or groups of tumors. Methylation of Rb2/p130 is frequent in sporadic retinoblastoma but not in the familiar one [27]. BRCA1 was found hypermethylated only in breast and ovarian carcinomas [26], whereas hypermethylation of the mismatch repair gene hMLH1 is restricted to the three sporadic tumor types characteristic of the hereditary nonpolyposis colorectal cancer syndrome: colorectal, endometrial, and gastric tumors with microsatellite instability [28]. Moreover, in any given tumor is possible to find simultaneous inactivation of several pathways by aberrant methylation compromising either cell survival or tumor progression genes. For example, the tumor may have disruption of cell cycle, DNA repair, and metastasis-related process by hypermethylation of $p16^{INK4a}$, hMLH1, and TIMP-3, respectively, whereas mammary tumor can accomplish similar objectives by silencing $p16^{INK4a}$,BRCA1 and CDH1 and a lung tumor affecting $p16^{INK4a}$, MGMT and DAPK [29]. Deregulation of matrix degrading metalloproteinase enzymes (MMPs) leads to increased extracellular matrix turnover, a key event in the local invasion and metastasis of many tumors. Expression of tissue inhibitor of metalloproteinase-3 (TIMP-3), a secreted protein bound to the extracellular matrix, antagonizes matrix metalloproteinase activity and has been shown to inhibit many aspects of tumor development and metastatic progression, including growth, angiogenesis and invasion [30]. Decreased TIMP-3 expression has been observed in a variety of tumor cell lines and has been associated with CpG island methylation. Aberrant methylation of TIMP-3 occurs in primary cancers of the kidney, brain, colon, breast and lung, but not in normal tissue and is particularly frequent, in renal cancer in which 78% of case has aberrant TIMP-3 methylation, with associated lack of protein expression [31]. Tumor-specific methylation of TIMP-3 may be a critical step during malignant progression. It is believable that loss of TIMP-3 may abrogate normal apoptotic programs, enhance primary tumor growth and angiogenesis, invasiveness and metastasis and possibly, therefore, contribute to all stages of malignant progression [32].

In addition to silencing as a result of mutations, loss of heterozygosity, or *classical* genetic events, the epigenetic modification symbolizes essential early events during carcinogenesis and tumor development. The reversion of these epigenetic processes restoring normal expression of tumor-suppressor genes has consequently become a new therapeutic target in cancer treatment. Aberrant patterns of epigenetic modifications will be, in a near future, crucial parameters in cancer diagnosis and prognosis.

2. Aberrant DNA Methylation and Drug Resistance of Tumors

One phenomenon common to both intrinsic and acquired resistance is aberrant alteration of gene expression in the drug-resistant tumor, which exceed in number respect to sensitive one. Although misregulation of gene expression has many origins, one such origin is *via* aberrant epigenetic regulation. As well as affecting disease progression, gene silencing has potential to influence resistance and clinical outcome following therapy. A number of recent studies suggest a direct role for epigenetic inactivation of genes in determining tumor chemosensitivity [33-35]. Aberrant deregulation of cell growth has traditionally been viewed as the major underlying mechanism for tumor formation; however, it is becoming increasingly clear that cellular changes that lead to inhibition of apoptosis play an essential role in tumor development and cellular drug response [36]. Many cancer chemotherapeutic drugs activate apoptotic mechanisms of tumor cell death, suggesting that factors that impair programmed cell death contribute to the resistance of tumor cells to cytotoxic drug treatment. Because the death of tumor cells induced by chemotherapy and radiotherapy is largely mediated by activation of apoptosis, inhibition of apoptosis will make tumor cells resistant to anti-tumor treatment. Numerous works were devoted to the identification of the cell death pathways that were triggered in tumor cells following drug or radiation therapy [37]. The role of p53 as a mediator of cytotoxicity of chemotherapeutic drugs is well accepted and the loss of p53 function is a common feature in human tumors contributing both to aggressive tumor behavior and to therapeutic resistance. p53 plays a pivotal role in modulating the cell responses to various sources of damage and stress by controlling the transcription of a large number of genes required for the apoptotic response [38]. Given that p53 plays a crucial and multivariate role in controlling cell growth and survival, it is not surprising that it has been found inactivated in the majority of cancers. Numerous genetic and epigenetic changes in the cancer cell may contribute to inactivate the function of oncosupressor genes thus leading to select a drug resistance phenotype. However, with the recognition of important roles for both p53 and its recently described paralog p73 in mediating the activity of anti-cancer drugs, there has been increasing recognition that cellular resistance to such agents can arise from failure of p53 family member signaling [39]. Whereas mutations of p53 occur commonly in tumors, inactivating mutations in the coding sequence of p73 are uncommon in human cancer. There are examples, particularly in hematological neoplasm, in which methylation-dependent silencing of p73 appears to occur in clinically aggressive cases and to correlate with poor response to therapy [40]. Another example is the osteosarcoma, which bears

mutations of both p53 and pRb1/p105 tumor suppressor genes, nevertheless these cells could still undergo apoptosis upon drug treatment in a p73-dependent pathways [41]. However, in total 30-40% of human osteosarcoma showed resistance after a few courses of chemotherapeutic treatment, which determinate the failure of therapy. It has been recently reported that multi-drug resistant (MDR) osteosarcoma failure the apoptotic response induced by chemotherapeutic drugs by maintaining repressed p73 transcription in a methylation-dependent manner, suggesting that epigenetic events occur to select a MDR osteosarcoma variants [39]. Apaf-1 represents another gene whose methylation may leads to increased resistance to chemotherapy. Metastatic melanoma is a very aggressive cancer that fails to respond to conventional chemotherapy which often lacks Apaf-1, a cell-death effector that acts with cytochrome c and caspase-9 in inducing p53-dependent apoptosis. Loss of Apaf-1 expression can be recovered in melanoma cell lines by treatment with the methylation inhibitor 5-aza-2-deoxycytidine thus suggesting that in these tumor histotype Apaf-1 is methylated. Apaf-1-negative melanomas are invariably chemoresistant and are unable to execute a typical apoptotic program in response to p53 activation. Restoring physiological levels of Apaf-1 through 5-aza-CdR treatment markedly enhances chemosensitivity and rescues the apoptotic defects associated with Apaf-1 loss [42]. Methylation of other members of Apaf-1 apoptotic network and caspase cascade can influence apoptosis and hence chemosensitivity. For example, the gene encoding for caspase-8 is frequently methylated in tumors and again demethylating agents can induce gene re-expression, increased apoptosis and chemosensitization [43]. The hMLH1 (mutL homologue 1) protein, part of the human DNA MMR (mismatch repair) system, has been shown to be important in determining sensitivity to a number of important chemotherapeutic agents [44]. The majority of sporadic colon [45], gastric [46] and endometrial [47] cancers are hMLH1 deficient and exhibit promoter hypermethylation of this gene. Experimental evidence suggests that for some cytotoxic drugs, DNA MMR proteins provide a link between recognition of DNA damage and downstream effectors of an apoptotic response, such as p53 and p73 [48]. Loss of DNA MMR proficiency results in resistance *in vitro* to a number of clinically important anticancer drugs, including cisplatin and doxorubicin [49], and it has been associated with selection for drug-resistant breast and ovarian tumors during chemotherapy [50]. Reintroduction of the hMLH1 gene into the hMLH1 null mouse cells leads to sensitization to DNA damaging agents [51], supporting a direct involvement of DNA MMR in drug sensitivity and providing evidence that re-expression of hMLH1 can partially overcome MMR-related drug resistance. In ovarian cancer, a higher frequency of hMLH1 promoter methylation is observed in post chemotherapy tumors, suggesting that aberrant hMLH1 promoter hypermethylation occurs in selecting clones that acquired drug resistance after chemotherapy treatments. Re-expression of hMLH1 by treatment with the demethylating agent 5-Aza-CdR results in sensitization of resistant variants to cisplatin *in vitro* [52]. The observation that demethylation of the *hMLH1* gene promoter results in drug sensitization *in vitro* raised the exciting possibility that MMR-related drug resistance could be overcome clinically by demethylating agents. Several genes in ovarian cancer, including tumor suppressors and genes involved in apoptotic pathways related to chemotherapeutic action are down regulated by epigenetic mechanisms. Another tumor suppressor found to be methylated and silenced in ovarian cancer is the gene encoding RASSF1A protein [53]. RASSF1A has been reported to bind to tubulin and stabilize microtubules [54] and it is possible hypothesize that this protein might ''assist'' chemotherapeutics such as paclitaxel in mediating prevention of spindle assembly. Re-expression of RASSF1A by epigenetic drugs could, conceivably, resensitize resistant tumors to such taxanes. In ovarian cancer, drug resistance induced by epigenetic events likely contributes to chemoresistance at several possible positions in drug response pathways. Epigenetic down regulation of Apaf-1, hMLH1, RASSF1A, $p16^{INK4a}$ and (possibly) p73 likely contributes to resistance, as well as the up regulation of FancF and MDR-1 in ovarian cancer [33].

While methylation of pro-apoptotic genes could lead to drug resistance, methylation of DNA repair genes, drug metabolisms and detoxification genes (GSTp1, MDR1) during tumor development might lead to drug sensitivity. The multidrug resistance phenotype (MDR) is associated whit expression of the MDR1 gene that encodes the drug transporter P-glycoprotein. P-gp protein is a member of the protein family, named ATP-binding cassette transporter proteins (ABC). These transmembrane proteins enhance drugs efflux in an energy dependent manner. The human ABC gene family consists of 48 members [55], and they are present in virtually every cell and play central role in physiology, working as efflux pumps in tissue defence [56]. Collectively, these proteins are capable of transporting a vast and chemically diverse array of toxicants. The overexpression of ABC transporters such as P-glycoprotein, the multi-drug resistance associated protein 1 (MRP1), MRP2 and the breast cancer resistance protein (BCRP) is associated with increased efflux of chemotherapeutic drugs such as anthracyclines, epipodophyllotoxins and vinca-alkaloids, and this can result in the so called multi-drug resistance (MDR). The overexpression of P-gp has been associated with resistance to a wide range of anticancer drugs. P-glycoprotein confers cross-resistance to unrelated drugs that differ widely with respect to molecular structure and target specificity, including many natural product agents (e.g., paclitaxel, vincristine, and doxorubicin) as well as new targeted anticancer agents (e.g., Gleevec) [57].

The precise mechanism of transcriptional regulation has been unclear due to the complex regulatory nature of the P-gp gene. It has become increasingly apparent that *trans*-activation or genetic amplification is by no means the only mechanism of activation. Consequently, alternative pathways have received more attention in the area of epigenetic events to help in explaining transcriptional competence at a higher level of organization. It has been shown an inverse correlation between functional gene expression and MDR1 promoter methylation, which would be protected by methylation in MDR tumors. The MDR1 promoter is differentially methylated in drug sensitive (non-expressing MDR1) and resistant (expressing MDR1) cells, predicting that the promoter is hypomethylated in the drug resistant disease and hypermethylated in the sensitive one [35].

The expression level of MGMT, a DNA repair enzyme, is proportional to the resistance of tumor cells to chemotherapic drugs such as cyclophosphamide. It has been shown that glioma cells with reduced MGMT expression are more sensitive to alkylating agents [58]. Hypermethylation of the MGMT promoter also correlate with increased survival of patients with diffuse large B-cell lymphoma after chemotherapy, which included cyclophosphamide [29]. FancF is crucial for the activation of a DNA repair complex containing BRCA1 and BRCA2 and the inactivation in this pathway results to a decreased ability to repair DNA damage and an increased susceptibility to develop cancer [59]. Methylation of the Fancf gene has been observed in ovarian cancer [60], acute myeloid leukemia [61], lung and head and neck cancers [62], and demethylation of Fancf promoter region, after treatment with 5-aza-2-deoxycytidine, reduced sensitivity towards cisplatin in these cell line models [60]. A two-step model for the role of the Fancf gene in tumorigenesis and acquired chemoresistance has been proposed [60]. According to this model, epigenetic inactivation of Fancf is an early event in tumor progression but subsequently chemotherapy selects cells in which the Fancf methylation was reversed and which, therefore, display higher resistance to platinum-based chemotherapy.

It has been proposed that the opposing processes of regional hypermethylation and global hypomethylation coexists in the same cell and that are two independent processes. Both global hypomethylation and regional hypermethylation confer a selective advantage upon cancer cells by targeting different sets of genes with opposing roles in cellular transformation. Regional hypermethylation targets the silencing of genes, which suppress tumorigenesis while global hypomethylation probably targets activation of genes, which are required for different stages of the transformation process.

Numerous correlations were obtained between tumor cell line chemosensitivity and the expression or mutation of specific genes. However, considering the effect of factors in isolation is insufficient because chemosensitivity involves multiple interacting factors that contribute to the overall response. There is probably no single gene or small group of genes determining the sensitivity or the resistance to a given anticancer drug. More likely, the concerted action of several genes with suppressive or permissive action eventually determines the activity of the drug towards the tumor cell. Each cancer cell represents a different pattern of drug-resistance gene expression even within cells clonally derived from the same cancer, and may be expected to exhibit a considerable amount of heterogeneity with respect to drug resistance [57].

Epigenetic changes are an important feature of cancer cells with acquired drug-resistant phenotype and may be a crucial contributing factor to its development. Finally, deregulation of similar pathways may explain the existence and provide mechanism of cross-resistance of cancer cells to different types of chemotherapeutic agents.

DNA HYPOMETHYLATING AGENTS AND HISTONE DEACETHYLASE INHIBITORS AS THERAPEUTIC TOOLS IN CANCER

The fact that human diseases, including cancer, can have an epigenetic etiology has encouraged the development of a new therapeutic option that might be termed "epigenetic therapy". Unlike genetic changes, the epigenetic changes in cancer are potentially reversible. Reactivation of the silencing associated with promoter methylation for critical genes would be a highly desirable goal for reversing many aspect of the cancer cell phenotype. Because methylation causes the inactivation of numerous genes that are important in the development of most of all tumors types, inhibition of DNA methylation and consequent re-activation of these genes is an attractive avenue for the development of novel therapeutics.

1. Epigenetic Therapy – A New Development in Pharmacology

Epigenetic therapy, based on use of drugs able to correct epigenetic defects, is a concept that has taken 15 years to develop and now represents a new branch of pharmacology.

The epigenetic therapy developed from the idea of treating cancer not by killing the cells but by changing their gene expression profile. Growing evidence indicates that epigenetic defects in cancer patients can compromise the response to chemotherapy treatment and that it exists a unique profile of promoter hypermethylation for each human cancer in which some methylated genes are shared and other are tumor-type-specific. On the bases of these finding, it is now emerging a new branch of personalized medicine, pharmacoepigenetics, which studies the patients response to treatment based on individual epigenetic differences, with the aim of improving the individual response to therapy [63]. The potential reversibility of epigenetic abnormalities has encouraged the development of pharmacologic inhibitors of DNA methylation and histone deacetylation as anti-cancer therapeutics. Two classes of epigenetic drugs are under investigation to treat cancers: DNA methyltrasferase (DNMT) inhibitors target DNA hypermethylation, and histone deacetylase (HDAC) inhibitors target histone deacetylation (Table **1**).

Among the DNMT inhibitors, the nucleoside analogous inhibitors 5-azacytidine (5-Aza-CR, Azacitidine, Vidaza) and 5-aza-2'-deoxycytidine (5-Aza-CdR, Decitabine, Dacogen) are the most extensively studied and they were recently approved by the U.S. Food and Drug Administration (FDA) as chemotherapeutic agents for myelodysplastic syndromes (MDS). These nucleosides are analogous of cytosine and are phosphorylated to the deoxynucleotide triphosphate and then incorporated into replicating DNA in place of natural base cytosine. Once incorporated into the DNA, a complex is formed with active sites of DNMTs, thereby covalently trapping these enzymes [64]. This results in the depletion of active enzymes and the demethylation of DNA after several cell divisions. A difference between 5-azacytidine and 5-aza-2′-deoxycytidine is that the first is partly incorporated into RNA, thereby interfering with protein translation, while 5-aza-2′-deoxycytidine is incorporated only into DNA, causing more efficient inhibition of DNMTs. The major disadvantages of 5-azacytidine and 5-aza-2′-deoxycytidine are their instability in neutral aqueous solution and their myelotoxicity resulting in cytopenia. More stable cytidine analogs, such as 5,6-dihydro-5-azacytidine and 5-fluoro-2′-deoxycytidine have been developed. Both these drugs are undergoing Phase

Table 1. Classification of Epigenetic Drugs According to Potential Therapeutic Use and Clinical Trials Phase

Inhibitor	Alternate Name	Use	State of Development
DNA Methyltransferase Inhibitors			
Nucleoside Analogue Inhibitors			
5-azacytidine	Azacytidine, Vidaza	MDS Solid tumors Leukaemia	FDA approved for clinical use Phase II/III Phase II/III
5-aza-2'-deoxycytidine	Decitabine, Dacogen	MDS Leukaemia	Phase II/III Phase II/III
Zebularine	---	Urinary bladder cancer	Preclinical
Arabinosyl-5-azacytidine	Fazarabine	Leukaemia	Phase I/II
5-6-dihydro-5-azacytidine	DHAC	Melanoma Solid tumors	Phase I/II Phase I/II
5-fluoro-2'-deoxycytidine	FdCyd	Cancer	Phase I/II
Non-nucleoside Analogue Inhibitors			
Epigallocatechin-3-Gallate	EGCG	Photocarcinogenesis Cancer of cervix	Preclinical Preclinical
Procainamide	---	Prostate cancer	Preclinical
Procaine	---	Breast cancer	Preclinical
Antisense oligonuclotides			
MG98	Dnmt1 Antisense	Renal Carcinoma	Phase II
Small Molecule			
RG108	---	---	Preclinical
HDAC Inhibitors			
Hydroxamates			
Suberic Bishydroxamic Acid	SBHA	---	---
Suberoylanilide Hydroxamic Acid	SAHA, vorinostat, Zolinza	Solid Tumors Leukaemia, MDS	Phase I/II Phase I/II
Trichostatin A	TSA	Breast cancer Ovarian cancer	Preclinical Preclinical
Cyclic hydroxamic-acid-containing peptide 1	CHAP1	---	---
LAQ824	NVP-LAQ824	Solid Tumors Hematologic Diseases	Phase I Phase I
Oxamflatin	---	---	---
PXD101	---	---	Phase I
Suberoyl-3-aminopyridineamide hydroxamic acid	Pyroxamide	Gynecologic cancer	Phase I
Cyclic Tetrapeptides and Analogues			
Apicidin	---	Leukaemia	Preclinical
Depsipeptide	---	Leukaemia Melanoma Colon cancer	Phase I/II Preclinical Preclinical
FK228	---	Leukaemia	Phase I/II
FR901218	---	Leukaemia	Phase I/II
Trapoxin A	TPX A	---	---

Table 1. contd….

Inhibitor	Alternate Name	Use	State of Development
HDAC Inhibitors			
Aliphatic Acids (Short-Chain Fatty Acids)			
Butyrates	Phenylbutyrate, Buphenyl	MDS, Leukaemia Urea cycle disorders	Phase I FDA approved for clinical use
Valproic acid	Depakote, Depakene	Bipolar disorder Cervical cancer, MDS	In routine use Phase I/II
Benzamides			
MS-275	---	Solid Tumors, Lymphoma	Phase I
CI-994	N-Acetyl-dinaline	Solid Tumors	Phase I/II
MGCD0103	---	Leukemia MDS Solid tumors	Phase I/II (combined with Aza-cytidine)

I and II studies. For 5-Fluoro-2′-deoxycytidine has been observed some toxic effect probably due to its metabolites [65, 66]. Zebularine is a novel cytosine analogous, which is very stable, less toxic and can be orally administered. However, it has the disadvantage of being much less potent than5-azacytidine and decitabine and needs to be administered in higher doses [67]. Although these properties make zebularine a promising candidate for cancer treatment, the requirement of higher concentrations (up to 1 g/kg body weight in the mouse model) in comparison with 5-aza-2′-deoxycytidine has important consequences for the clinical potential [68]. The myelotoxicity of nucleoside analogs, as sociated with their incorporation into DNA, resulted in the search for non-nucleoside DNMT inhibitors. A group of non-nucleoside DNMTs inhibitors that are not incorporated into DNA because of structural differences from cytosine, has been identified.

Procainamide and procaine inhibit DNMTs by perturbing interactions between the protein and its target sites. Both these drugs are undergoing preclinical trials because of their growth-inhibitory effects in prostatic and breast cancers [69, 70].

Another non-nucleoside analogous is epigallocathechin-3-gallate, a natural product derived from green tea, which has shown to inhibit DNMT activity by binding to and blocking the active site of human DNMT [71]. RG108 is a novel small molecule that blocks the DNMT active site. Intriguingly, it causes demethylation and reactivation of tumor suppressor genes, but does not affect methylation of centromeric satellite sequences. These characteristics make RG108 particularly useful for new drug developmen [72]. Most DNMT inhibitors are not specific for a particular DNMT, which may result in unfavourable toxicity. Therefore, new compounds with specificity for a particular DNMT are being developed. Antisense oligonucleotides, such as MG98, that are complementary to mRNA for human DNMT1 and able to block the translation of DNMT1, are undergoing clinical trials [73].

As regard HDAC inhibitors, many naturally occurring and synthetic histone deacetylase inhibitors have been characterized and some of them displayed anticancer activities in preclinical studies. These compounds are structurally heterogeneous, and have been classified according to their chemical nature and mechanism of inhibition such as their affinity for the HDACs of classes 1, 2, 4. Among HDAC inhibitors, the hydroxamic acids are very potent but reversible HDAC inhibitors, that bind more strongly to the HDAC catalytic site [74]. Among these compounds is trichostatin A (TSA), originally developed as an antifungal agent, which is active at nanomolar concentrations [75]. Other hydroxamic acids are suberoylanilide hydroxamic acid (SAHA), which has been recently approved for therapy of cutaneous T-cell lymphomas, pyroxamide, oxamflatin, PXD101, NVP-LAQ824 and LBH589. The hydroxamate Scriptaid is a novel synthetic HDAC inhibitor with a relatively low toxicity. The cyclic hydroxamic-acid containing peptide (CHAP) compounds are built from TSA and cyclic tetrapeptides and inhibit HDACs at nanomolar concentrations [76].

Other classes of HDAC inhibitors are short chain fatty acids (SCFA), benzamides, epoxyketone and non-epoxyketone containing cyclic tetrapeptides, and hybrid molecules. SCFA, although widely used (especially valproic acid) and clinically efficacious, have weak HDAC inhibition constants.

The short-chain fatty acids phenylbutyrate and valproic acid are relatively old drugs that have been used for non-oncological uses and recently shown to have activity as HDAC inhibitors [74]. These compounds possess an acyl group, which contacts the catalytic HDAC zinc ion but cannot make significant contact with the catalytic pocket due to their very short side chains. Therefore, phenylbutyrate and valproic acid act as HDAC inhibitors at relatively high concentrations. A third class of HDAC inhibitors is the cyclic tetrapeptides, including depsipeptide (FK-228, FR901228), apicidin and trapoxin. Depsipeptide is a pro-drug that is activated by reduction upon cellular uptake and inhibits class I HDACs, although the exact mechanism of inhibition remains unknown [77]. Apicidin is a reversible HDAC inhibitor at low nanomolar concentrations, bearing an alkylketone residue that is supposed to chelate the catalytic HDAC zinc ion [78]. Trapoxin is closely related to apicidin, and irreversibly inactivates HDAC by covalent interaction between its epoxide group and the HDAC catalytic site.

The benzamides are a structurally diverse fourth class of HDAC inhibitors including MS-275 (Benzamidine), CI-994 and MGCD0103 which act by binding the active zinc in the HDAC catalytic site. The synthetic HDAC inhibitor MS-275 inhibits HDAC at micromolar concentrations. CI-994 (*N*-acetyl dinaline) is a relatively weak HDAC inhibitor and the mechanism of its action is still unknown. It inhibits histone deacetylation, but not by inhibiting HDAC activity. Benzamides, like MS-275, and cyclic peptides, like depsipeptide, have been studied in numerous clinical trials and demonstrated low toxicity and activity in solid and haematological neoplasms [76, 79].

2. Insights on the Clinical Trials with Hypomethylating and Deacetylating Agents

It is clear from *in vitro* and preclinical studies that the clinical application of reversing epigenetic aberrations in tumor cells, called epigenetic therapy, is a promising strategy for cancer treatment. As previously described, many agents have been discovered that inhibit DNA methylation and histone deacetylation. However their therapeutic value will be established by ongoing clinical trials.

Some drugs have been around for decades and have been used as chemotherapeutics for cancers and other disease, other are of new generation and are under investigation to evaluate their possible use in epigenetic therapy. Currently, although some DNMT inhibitors received approval to be used in Phase I and II clinical trials, no HDAC inhibitors are approved for cancer therapy except for suberoylanilide hydroxamic acid, or SAHA (Zolinza), which has been submitted as new drug application for treatment of cutaneous T-Cell lymphoma [80-82]. HDAC are approved for diseases other than cancers, including phenylbutyrate (Buphenyl) for urea cycle disorders and valproic acid (Depakote) for seizures. Both these short chain fatty acids are under evaluation as potential epigenetic cancer drugs [79]. Sodium butyrate and sodium phenylbutyrate were the first HDAC inhibitors to be tested in cancer patients despite the limitations of low potency and lack of specificity of these compounds [83, 84]. Phase I clinical and pharmacokinetic studies of sodium phenylbutyrate has been evaluated in AML and MDS [85], as well as in solid tumor malignancies [86, 87]. Millimolar or high micromolar peak plasma concentrations can be achieved following intravenous (iv) and oral administration. Prolonged iv infusions of sodium phenylbutyrate maintain constant circulating concentrations of the drug at potentially therapeutic levels but are complicated by somnolence and confusion [88]. Sodium phenylbutyrate is well absorbed from the gut but very large oral doses of several grams per day are needed to achieve biologically active plasma concentration of 0,5 mM. Another short chain fatty acid HDAC inhibitor, valproic acid, has been used as an antiepileptic drug for many years but only recently it was shown to inhibit HDAC at millimolar or high micromolar concentrations [89]. Phase I and II clinical trials for evaluating it as an anti-cancer agent have been recently reported [90, 91]. Although there is a wealth of clinical experience with valproic acid, it suffers from the same limitations as the other short-chain fatty acid HDAC inhibitors, namely low potency and specificity. There are metabolic and other potentially serious dose-related toxicities that preclude administration of valproic acid at potentially therapeutic anticancer doses. There are other HDAC inhibitors, the bicyclic depsipeptides, in early phase clinical development as anticancer drugs for the treatment of solid and hematological cancers [92]. FR-901228 is one of these compounds to enter clinical trials and is now in phase II development [93, 94]. Preclinical studies in rodents showed that peak plasma levels in excess of those predicted to be therapeutic *in vitro* could be achieved with single iv or oral doses of FR-901228 and could be sustained with iv infusion [95]. Preclinical studies predicted significant cardiac and catheter-side related toxicity but FR-901228 was well tolerated in patients with relatively mild hematological (neutropenia, thrombocytopenia) and non-hematological (nausea/vomiting, fatigue, ECG change, hypocalcemia) toxicity [96]. Phase I and II studies of FR-901228 confirmed efficacy in peripheral and cutaneous T-cell lymphomas and in chronic lymphocytic leukemia [94]. Phase I trials with depsipeptide have shown encouraging results, especially for patients with cutaneous T-cell lymphoma. Phase II studies are ongoing to establish their efficacy in a range of solid and hematological malignancies [92].

Among the organic hydroxamic acids, the tricostatin A was the first one tested in laboratory but it has limited use because its extremely short half-life, and other hydroxamic acids have been identified and developed for clinical use [97]. Suberoylanilide hydroxamic acid, SAHA, is the most advanced in development and is currently under testing in both intravenous infusion and oral administration. Encouraging results were obtained in Phase I and II clinical trials for patients with both hematologic and solid tumors [98]. A phase I study was conducted to evaluate the safety and activity of oral vorinostat (SAHA) 100-300 mg administered twice- or thrice-daily for 14 days followed by 1-week of rest. Patients with relapsed or refractory leukemias or myelodysplastic syndromes (MDS) and untreated patients who were not candidates for chemotherapy were eligible. The maximum tolerated dose (MTD) was 200 mg twice-daily or 250 mg thrice-daily. Dose-limiting toxicities were fatigue, nausea, vomiting, and diarrhea [80]. Currently, Phase II study of oral vorinostat (SAHA) for refractory cutaneous T-cell lymphoma [81] and Phase III clinical trials in patients with malignant pleural mesothelioma and diffuse large B-cell lymphoma are ongoing. While other hydroxamic acids such as LAQ824 have entered clinical trials, there is little data published about their toxicities or response observed to date. Among the favorable aspects of hydroxamic acids are their tolerability and oral absorption while one of the main limitations is their short half-life.

Among the benzamides drugs, a phase I study of MS-275 has been performed in patients with advanced solid tumors or lymphoma [99]. In this study a cohort of patients treated with MS-275 daily showed severe toxicity, including elevated liver enzymes, hypophosphatemia, hypoalbuminemia and pleural effusion. Pharmacokinetic analysis showed that MS-275 had a much longer half-life than expected. The trial was revised to evaluate the true half-life and the MS-275 was administered either once every 7 or 14 days. The observed toxicities included fatigue, nausea, vomiting, hypoalbuminemia, anxiety, dyspepsia, anemia, fever and dysgeusia. Currently, Phase II clinical trials is undergoing in patients with advanced and refractory solid tumors and lymphoma

[100]. Phase I clinical trial of chronic administration of acetyldinaline CI-994 was recently published [101], where orally daily administration for 8 weeks was done in patients with non-small lung cancer. The observed maximum tolerated dose was 8mg/m2/d and dose limiting toxicities were neutropenia and thrombocytopenia with other effect reported as leukopenia, nausea, vomiting, diarrhea, fatigue and anorexia. In this study it was observed an adaptation phenomenon of the platelet count, which rebounded after the first month of therapy. Phase II studies have been also conducted in patients with advanced non-small cell lung cancer, metastatic renal cell carcinoma and advanced pancreatic cancer [102, 103]. However, Phase II of its activity as monotherapy have proved disappointing and further phase II trials of CI-994 in combination with chemotherapeutic agents as capecitabine, gemcitibine, or carboplatin with paclitaxel are underway [104].

The favorable aspects of the benzamides include their tolerability and oral absorption. While the unexpectedly long half-life of these compounds may be a favorable attribute, the best schedule has yet to be determined.

Many questions still remain unanswered regarding the optimal evaluation and utilization of HDAC inhibitors for cancer prevention and treatment. It is still unclear whether the clinical effects of HDAC inhibitors are the results of alterations of histone acetylation patterns or of increased non-histone proteins acetylation, which determines changes in growth regulatory pathways. Due to existence of many different HDACs it is crucial to understand the specificity of the existing HDAC inhibitors as well as to develop selective inhibitors that target individual enzymes.

Many agents have been discovered that inhibit DNA methylation and with respect to HDAC inhibitors the value of these compounds as efficient anti-cancer drugs is well established. 5-Azacytidine and 5-Aza-2'-deoxicitidine represent the most prominent DNMT inhibitors used in clinical practice [105] and that they received the FDA approval.

The nucleoside inhibitors, 5-azacytidine (Vidaza) and 5-Aza-2'-deoxicitidine (decitabine), have been tested in phase I and II trials against many forms of cancer. The dose-limiting toxicity for both is myelosuppression, and the most commonly reported non-hematologic adverse effect was nausea and vomiting. In lung cancer patients, toxicity was both dose and schedule dependent, with decitabine being less myelosuppressive when the same dose was administered over a shorter treatment period than over a longer period [106]. At cytotoxic doses, decitabine was active against leukemia and myelodysplastic syndromes, but only limited activity with these schedules and doses was observed against solid tumors [107, 108]. More recently Phase II and III clinical trials have been conducted for the treatment of myelodysplastic syndrome (MDS) and chronic myelogenous leukemia by using low-dose 5-azacytidine or decitabine and significantly higher response rates were reported in the patient group treated with demethylating agents as compared with the group receiving supportive care only. Also the quality of life was significantly improved in the group that received aza-nucleoside treatments [109, 110]. These results led to recent FDA approval of 5-azacytidine (Vidaza) for treatment of all MDS subtype and to the fast-track status of Decitabine (Dacogen) for MDS [111]. Because decitabine also showed promising response rates for other leukemia [112, 113], it might be effective for other tumor types. However, the activity of decitabine in solid tumor remain unclear, although prolonged disease stabilization has been reported in patients with lung cancer, prostate cancer and other thoracic malignancies [106, 114, 115]. Although these demethylating agents have shown clinical benefit, there are several pitfalls regarding their clinical application. Their specificity on DNMT inhibition, reversal methylation and gene reactivation is not entirely clear. Many of nucleoside demethylating drugs are not specific for particular DNMT or gene and some of them showed high toxicity mediated primarily by covalent trapping of DNA methyltrasferase rather than DNA methylation [116]. Toxicity, a central problem in interpretating clinical data, might be reversed by optimizing treatment schedules e.g. giving lower doses over longer time periods [110] as well as by developing non-nucleoside inhibitors which may be less toxic because they are not incorporated into DNA. Non-nucleoside inhibitors such as EGCG, RG108 and procaine are now under evaluation in preclinical and clinical studies [117-119]. Also new compounds with specificity for particular DNMT might reduce nonspecific effects and hold promise for a more targeted approach towards methylation. Of these compounds, MG98 is currently being tested in Phase II clinical trial [120].

Targeting epigenetic gene regulation may require a combination of chromatin modifying agents. Recently, on the base of a multitude of experimental results, many epigenetic investigators believe some of these drugs might work better together than individually. Some HDAC inhibitors have been definitively shown to be synergistic with DNMT inhibitors in reacting gene expression *in vitro* and therefore there is the possibility that they will be clinically synergistic also, but the verdict is still out on that. Preclinical and Phase I/II clinical studies of DNA methyltransferase and histone deacetylase inhibitors have yielded encouraging results, especially against hematologic malignancies [121]. It has been reported clinical trial in which demethylating agent combined with HDAC inhibitor was administered to patients with hematological and solid tumors, achieving complete and partial remissions [122, 123]. Recently, Phase I study of decitabine in combination with valproic acid has been evaluated [124] as well as Phase I/II clinical trials of HDAC inhibitor MGCD0103 combined with azacytidine is actually evaluating in patients with either MDS or acute myeloid leukemia (AML) [125].

In conclusion targeting of cancer through demethylation and histone acetylation proves to be an exiting area of cancer therapy. At this time development of second generation inhibitors of DNA methyltrasferase and HDAC is in progress and their efficacy as anti-tumoral drugs will ultimately require evaluation in monotherapy and in combination. The manipulation of gene expression through epigenetic modifications heralds a new era of gene-targeted therapy and holds promise as both therapeutic and preventive strategy.

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